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A regulatory relationship between Tbx1 and FGF signaling during tooth morphogenesis and ameloblast lineage determination

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ABSTRACT

The *Tbx1* gene is a transcriptional regulator involved in the DiGeorge syndrome, which affects normal facial and tooth development. Several clinical reports point to a common enamel defect in the teeth of patients with DiGeorge syndrome. Here, we have analyzed the expression, regulation, and function of *Tbx1* during mouse molar development. *Tbx1* expression is restricted to epithelial cells that give rise to the enamel producing ameloblasts and correlates with proliferative events. *Tbx1* expression in epithelium requires mesenchyme-derived signals: dental mesenchyme induces expression of *Tbx1* in recombined dental and non-dental epithelia. Bead implantation experiments show that FGF molecules are able to maintain epithelial *Tbx1* expression during odontogenesis. Expression of *Tbx1* in dental epithelium of FGF receptor $2b^{-/-}$ mutant mice is downregulated, showing a genetic link between FGF signaling and Tbx1 in teeth. Forced expression of *Tbx1* in dental explants activates *amelogenin* expression. These results indicate that *Tbx1* expression in developing teeth is under control of FGF signaling and correlates with determination of the ameloblast lineage.

Introduction

Teeth are organs that develop as a result of sequential and reciprocal interactions between the oral epithelium and cranial neural crest-derived mesenchyme. These interactions gradually transform the tooth primordia into complex structures with various cell types, among which the epithelial-derived ameloblasts synthesize and secrete the organic components of the enamel (Mitsiadis, 2001; Ruch, 1995). Tissue recombination experiments have shown that the inductive capacity for mouse tooth formation resides in the epithelium until embryonic day 12 (E12), after which it shifts to the condensing mesenchyme (Mina and Kollar, 1987). The induced mesenchyme has the capacity to instruct a non-dental epithelium allowing it to participate in tooth formation (Kollar and Baird, 1969).

Four cell layers form the dental epithelium during odontogenesis: the inner dental epithelium (from which the ameloblasts originate), stratum intermedium, stellate reticulum and outer dental epithelium. The specification of these dental cell types may involve genes with restricted expression patterns to one or another cell-type during odontogenesis. While a number of genes are differentially expressed in dental cell populations (reviewed by Mitsiadis, 2001; Thesleff, 2006; Tucker and Sharpe, 2004), they are unlikely to play a formative role in cell fate specification because of their relatively late onset of expression. Our previous results suggest that molecules of the Notch signaling pathway may play a role in specifying dental cell-type identity (Mitsiadis et al., 1995a, 1997, 1998, 2005). Other candidate genes for controlling cell-type identity are the transcriptional regulator-encoding *T-box* genes, characterized by the presence of a highly conserved motif (T-box) that encodes a 180 amino acid DNA-binding domain (T-domain) (Bollag et al., 1994). *T-box* genes are expressed throughout development and seem to play an important role in the specification of different cell populations (Naiche et al., 2005). Mutations in human *T-box* genes cause pleiotropic developmental disorders affecting, among others, tooth development (Bamshad et al., 1997; Basson et al., 1997; Braybrook et al., 2001; Li et al., 1997; Meneghini et al., 2006; Naiche et al., 2005; Packham and Brook, 2003). *TBX1* is a candidate for the DiGeorge syndrome (Chieffo et al., 1997; Jerome and Papaioannou, 2001; Lindsay et al., 2001). Studies on teeth of patients with DiGeorge syndrome have shown enamel formation defects (Børglum-Jensen et al., 1983; Fukui et al., 2000).

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Here we present a systematic analysis of *Tbx1* expression during mouse odontogenesis. We show that expression remains confined to the proliferating epithelial components of tooth primordia and distinguishes a specific dental cell lineage (inner dental epithelium cells) that gives rise to the amelogenin-producing ameloblasts. Furthermore, we provide evidence that epithelial–mesenchymal interactions and FGF signaling are involved in the regulation of *Tbx1* expression during tooth development.

Materials and methods

Animals and tissue preparation

Swiss and C57Bl/6 mice were used at embryonic and early postnatal stages (embryonic day 10.5 to 18.5; E10.5–E18.5). The age of the mouse embryos was determined according to the appearance of the vaginal plug (day 0) and confirmed by morphological criteria. Animals were killed by cervical dislocation and the embryos were surgically removed into Dulbecco's phosphate-buffered saline (PBS). Dissected heads from mouse embryos were fixed overnight at 4 °C in 4% paraformaldehyde (PFA) in PBS. The generation and genotyping of $Fgfr2b^{-/-}$ mice has been described previously (De Moerlooze et al., 2000).

Probes and in situ hybridization

Digoxigenin-labeled (Boehringer Mannheim) and radioactive antisense riboprobes for mouse *Tbx1* (Chapman et al., 1996), and *amelogenin* were used. Whole mount *in situ* hybridization on explants and *in situ* hybridization on cryosections and paraffin sections were performed as previously described (Mitsiadis et al., 1995b, 1997).

Recombinant proteins and beads

Recombinant BMP2, BMP4 (1.12 mg/ml; Genetics Institute, Cambridge, Massachusetts), FGF2 (Boehringer Mannheim, Germany), FGF3 and FGF4 (British Biotechnology Products) proteins were used to preload affi-gel agarose beads (75–150 μm diameter; Biorad) and heparin acrylic beads (100–200 mesh/100–250 μm diameter; Sigma). The proteins were diluted with 0.1% bovine serum albumin (BSA) in PBS, pH 7.4, to concentrations 50–250 ng/μl per 5 μl per 50 beads. As a control, we used beads loaded with 0.1% BSA in PBS. Beads preloaded with BMPs, FGFs and BSA were either transplanted or placed on top of mandibles and dental epithelia explants, and after 20 h in culture the explants were fixed in 4% PFA, washed in PBS and finally stored in MeOH at −20 °C until analysis by whole mount in situ hybridization (for details see Mitsiadis et al., 1995b, 2003).

Mandible explants

Mouse mandibles were used for bead implantation and electroporation experiments. Mandibles were dissected in Dulbecco's PBS from the rest of the heads of E10.5 to E12.5 embryos and placed into a solution of Dulbecco's Modified Eagle Medium (DMEM; GibcoBRL) containing 20 units/ml penicillin/streptomycin (GibcoBRL). The first branchial arches were placed on top of 0.1 mm Millipore filters on stainless steel wire meshes (0.25 mm diameter wire; Goodfellows) in organ culture dishes (Marathon) containing media consisting of DMEM, 20% foetal calf serum (FCS; GibcoBRL) and 20 units/ml penicillin/streptomycin, as previously reported (Mitsiadis et al., 2003). The mandibles were cultured in a humidified atmosphere of 5% CO_2, 40% O_2 at 37 °C for the designated lengths of time. After the required period of culture, explants were fixed in 4% PFA in PBS overnight at 4 °C and processed for whole mount in situ hybridization.

Tissue recombination experiments

The lower first molars and non-odontogenic oral areas of E12.5 mouse embryos were used for tissue recombination (epithelium–mesenchyme) and bead implantation experiments. After dissection, the tissues were incubated for 5 min in 2.25% trypsin/0.75% pancreatin on ice and the epithelia were mechanically separated from mesenchyme in Dulbecco's Minimum Essential Medium supplemented with 15% FCS. Isolated epithelia were cultured as recombinants with isolated mesenchyme in various homo- and heterotypic combinations on a polycarbonate membrane (Nuclepore Corp.). Furthermore, isolated dental epithelia were recombined together (2–3 tissues), as previously reported (Mitsiadis et al., 1997), to avoid apoptosis occurring when individual epithelia are cultured. After 24 h in culture, the explants were fixed in 4% PFA and then treated as whole mounts. Other heterotypic recombinants were cultured for 3 to 7 days, and after fixation whole mount in situ hybridization and in situ hybridization on 14 μm cryosections were performed. In recombinants cultured for 7 days, the epithelia were separated from mesenchyme and then immediately fixed and processed for whole mount in situ hybridization. A total number of 18 tissue recombinants were used for these experiments.

Slice culturing

E13.5 mouse mandibles were dissected out and sliced using a McIlwain tissue chopper (Mickle Laboratory Engineering Co. Ltd) into frontal slices 250 μm thick. These were then separated out and the slices with tooth germs showing clear bud formation were kept for culturing. Slices were cultured on millipore filters supported on metal grids over medium. Medium consisted of DMEM supplemented with penicillin, streptomycin, glutamine and 10% FCS. The filters were coated in Matrigel basement membrane matrix (BD Biosciences) to provide support for the slices, and then a second layer of Matrigel was added on top of the slices. More than 35 slices were cultured in this manner at 37 °C/5% CO_2 for four days.

DiI labeling and fate mapping of dental cells

DiI is a lipophilic dye, which intercalates in the cell membrane marking small groups of cells. DiI (Molecular probes cell tracker CM-DiI, C-7000) was prepared in EtOH at 2.5 μg/μl. This stock solution was then diluted 1 to 9 in 0.2 M sucrose and warmed. DiI was injected by a mouth-controlled micropipette made from a 50 mm borosilicate glass capillary. Different positions of the bud-staged (E13.5) dental epithelium were labeled with DiI, and the explants were then cultured, as described above, until the early bell-staged tooth (E16.5–E17.5) could be identified by morphological criteria. The fate of the labeled cells was assessed in cultured dental tissues and after 8 mm paraffin sectioning. The transmitted light and fluorescence images were captured with a Zeiss Axioscope equipped with a CCD camera, and thereafter the transmitted light and fluorescence images were merged.

Electroporation and expression construct

Electroporation was performed as described previously (Mitsiadis et al., 2003). Briefly, gene constructs were introduced to the targeted area of 12 mouse mandibles using fine glass needles filled with a DNA solution in 1% carboxy methyl cellulose. Needles were connected to a syringe pump through a fine silicone tube. Tungsten microelectrodes of a micromanipulator were inserted into the epithelium and DNA introduced into the cells using an Electro-Square-Porator™ ECM 830 (Genetronics). The pIRES2-EGFP expression vector (Clontech) has a green fluorescent protein (GFP), which allows visualization of the targeting efficiency of the electroporation. Full-length coding fragments for human *Tbx1* were cloned into this vector (constructed by Dr Paris Ataliotis and kindly provided by Professor Peter Scambler, ICH, UCL) and electroporated. Following electroporation, one side of the mandible was GFP-positive, whereas the other side was GFP-negative and thereby served as an internal control. Another control consisted of the pIRES2-EGFP vector alone. Explants were cultured for 24 h before fixation in 4% PFA and processed for section ^{35}S *in situ* hybridization.

Cell proliferation analysis

Cell proliferation in dental tissues was analyzed by using a bromodeoxyuridine (BrdU) cell proliferation kit (Boehringer Mannheim). For the detection of cell proliferation *in vivo*, foster mothers were injected intraperitoneally with 5 mg/ml of BrdU in PBS (concentration: 50 mg/g body weight) 30 to 60 min before embryos were killed. BrdU-positive cells in teeth of E13 embryos were analyzed on 14 μm cryosections after staining with an anti-BrdU antibody. For the detection of cell proliferation *in vitro*, the dental explants were cultured for an additional 30 min with BrdU, according to the manufacturer's instructions. Whole mount immunohistochemistry was performed as earlier described (Mitsiadis et al., 1995b).

Results

Tbx1 expression in developing teeth

To determine the role of Tbx1 in odontogenesis, we first analyzed the expression pattern of the *Tbx1* gene during mouse molar development. Tooth initiation starts as a local thickening of the oral epithelium, which invaginates the underlying neural crest-derived mesenchyme and progressively acquires the characteristic bud, cap and bell configurations. We monitored the expression of the *Tbx1* gene in dental tissues from E11.5 to E18.5 mouse embryos (E11.5–E18.5). An intense hybridization signal was observed in dental epithelium during the tooth initiation (dental placode; E11.5) and bud (E12.5–E13.5) stages (Figs. 1A, B, 2A, G and 5A). No hybridization signal was detected with the sense probe at these or subsequent developmental stages (data not shown). At the bud stage, the signal was mainly detected in the epithelial layer that is adjacent to the condensed mesenchyme (Figs. 1A, B). At the cap stage (E14.5), the dental epithelium gives rise to the enamel organ; the outer and inner dental epithelia can be distinguished in the epithelial component of

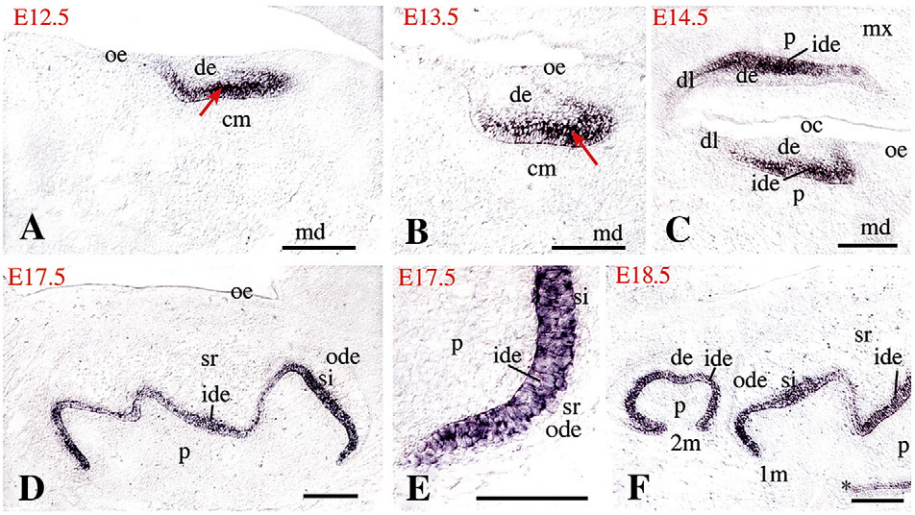

Fig. 1. Tbx1 expression in the developing mouse molar tooth. In situ hybridization on cryosections using a digoxigenin-labeled probe. (A, B) Sagittal sections through the head of E12.5 and E13.5 mouse embryos. *Tbx1* transcripts in dental epithelial cells (de; arrows). (C) Sagittal section through the head of an E14.5 mouse embryo. *Tbx1* expression in cells of the inner dental epithelium (ide). (D) Sagittal section through the head of E17.5 mouse embryos. *Tbx1* expression in cells of the inner dental epithelium. (E) Higher magnification of the previous figure showing *Tbx1* expression in the cervical loop area. (F) Sagittal section through the head of an E18.5 mouse embryo. *Tbx1* expression in cells of the inner dental epithelium. Faint expression in the epithelial root sheath (asterisk) of the first molar (1m). Note the strong *Tbx1* signal in the inner dental epithelium of the developing second molar (2m). Additional abbreviations: cm, condensed mesenchyme; de, dental epithelium; dl, dental lamina; eo, enamel organ; md, mandibular process; mx, maxillary process; oc oral cavity; ode; outer dental epithelium; oe, oral epithelium; p, dental papilla; si, stratum intermedium; sr, stellate reticulum. Scale bars: 200 μm.

the developing first molar. *Tbx1* transcripts were mainly detected in cells of the inner dental epithelium (Fig. 1C), whereas other cells of the enamel organ were not labeled. This expression pattern persisted during the bell stage (E17.5–E18.5) (Figs. 1D–F and 2F, L). The development of the second molar is delayed when compared with that of the first molar. Similarly to the first molar, *Tbx1* expression was restricted in inner dental epithelial cells of the second molar germ (Fig. 1F). Differentiation of inner dental epithelium cells into preameloblasts coincided with down-regulation of *Tbx1* expression (Fig. 1F), whereas *Tbx1* remained highly expressed in cells of the inner dental epithelium that are located in less developmentally advanced areas.

Lineage label of bud-staged dental epithelium

The expression pattern of *Tbx1* in the developing tooth suggests that it could be an early marker for cells of the inner dental epithelium/preameloblasts. To test this hypothesis, we monitored the movement of dental epithelial cells from the bud (E13.5) to the early bell stage (E16.5–E17.5) in cultured mandible slices using DiI injection. Growth factor reduced matrigel was used to keep the morphology of the slice during culture. When slices were cultured without matrigel the cells often moved out of the slice and the visualization of the tooth was difficult. Matrigel provides a physiologically relevant environment for tissue culture, and cells behave as *in vivo* conditions. Despite the slightly artificial nature of this culture system the use of matrigel and presence of wound healing do not detract from the detected cell labeling. More than 35 slices of E13.5 mandibles, which contain sectioned molar tooth germs with the typical bud configuration, were selected for culture. The slices were cultured for four days, when the tooth epithelium acquired the bell configuration (early bell-stage; E16.5–E17.5), to follow the fates of dental epithelial cells labeled with DiI and to compare their fate with *Tbx1* expression. During culturing, all explants retained their original morphology and the development of the tooth germs proceeded normally, passing from the bud stage (Figs. 2A, B, G, H) to the cap (Figs. 2C, I) and early bell (Figs. 2D, E, J, K) stages. We then labeled distinct parts of the exposed dental epithelium with DiI, which were located either proximally or distally to the epithelial–mesenchymal boundary. DiI was injected in the basal (Figs. 2A, B) part of the epithelial bud, which is in close contact with the condensed mesenchyme, as well as in the internal (median) part of the bud (Figs. 2G, H), far away from the epithelial–mesenchymal boundary. After labeling, slices were checked daily and photographed. In all cases no labeled cells moved out of the basal region to the internal (median) part of the developing tooth germs. The labeled cells remained as cohesive patches in the basal area of the tooth germ, which forms the inner dental epithelium layer, during the culture period (Figs. 2A–D). Because it was difficult to visualize individual DiI labeled cells in the slices and to analyze results in a detailed manner, slices were then fixed and serially sectioned after culturing. In these sections, labeling could be visualized at the single cell level (Fig. 2E). DiI labeled cells at the basal part of the tooth bud (Figs. 2A, B), which also express the *Tbx1* gene (Fig. 2A), were localized only in a part of the inner dental epithelium when the tooth germ reaches the early bell stage (Figs. 2D, E). Similarly, DiI labeled cells at the median part of the tooth bud (Figs. 2G, H), where *Tbx1* is not expressed (Fig. 2G), could only be seen in cells of the stellate reticulum and outer dental epithelium at the early bell stage (Figs. 2J, K). The present findings show that in dental epithelium the various cell populations do not intermingle and they maintain their initial identity: cells of the basal part of the tooth bud give rise only to cells of the inner dental epithelium, which express *Tbx1* (Figs. 2F, L), while cells of the median part give rise to cells of the stellate reticulum, which are not expressing *Tbx1* (Figs. 2F, L).

Requirement of dental mesenchyme for Tbx1 expression in epithelium

The presence of *Tbx1* transcripts in epithelial cells that are in proximity to the dental papilla mesenchyme (inner dental epithelium cells) suggests that *Tbx1* expression may be controlled by mesenchyme-derived signals. To test this possibility we recombined dissected epithelial and mesenchymal tissues from dental and non-dental regions (e.g. aboral epithelium, palate, lip) (Figs. 3A, B, E). Recombinations were carried out at E12.5, a time when the odontogenic potential has been transferred from the epithelium to the mesenchyme (Mina and Kollar, 1987). *Tbx1* expression was examined by *in situ*

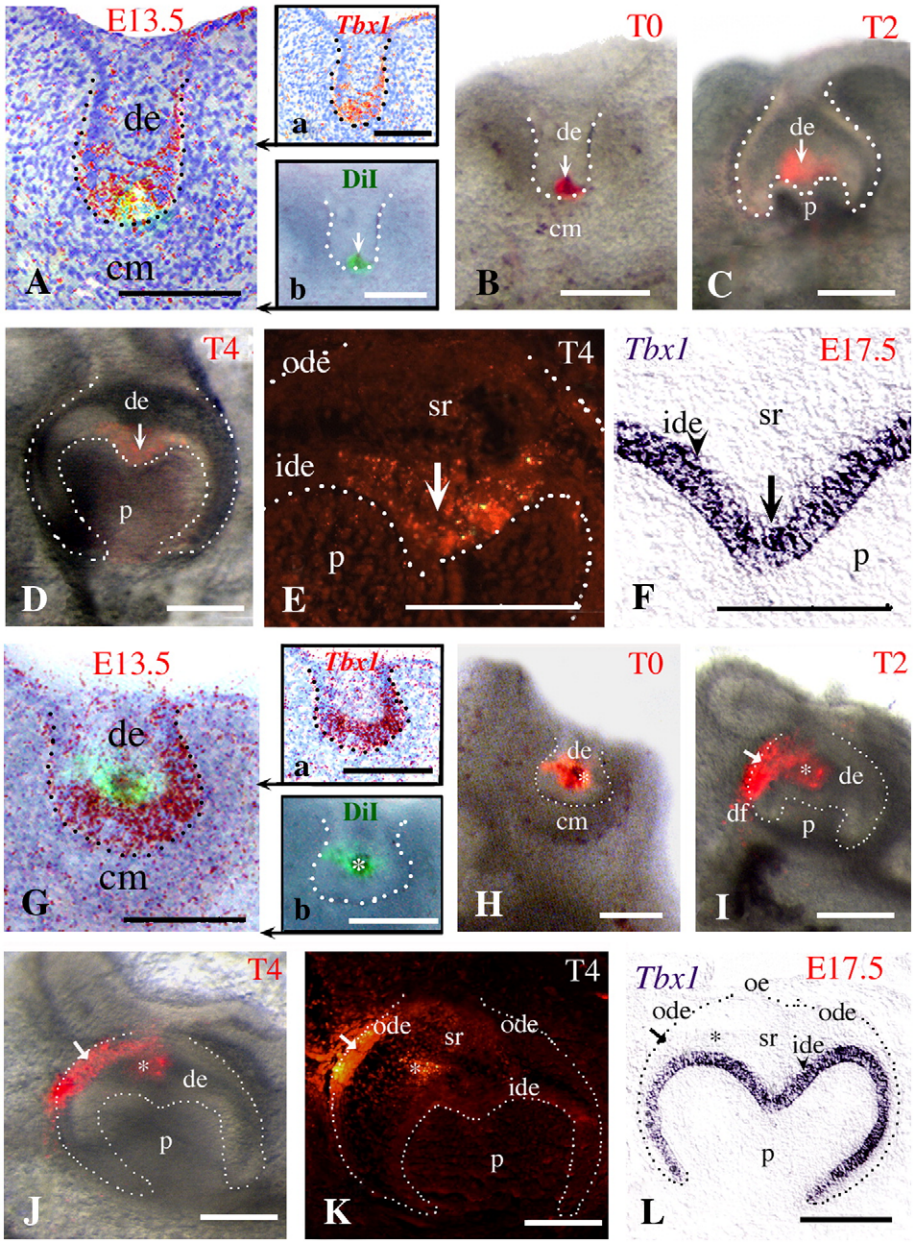

Fig. 2. DiI labeling of the developing lower molar germ in slice culture. (A, B, G, H) DiI detection immediately after labeling (T0) at the bud stage. (C, I) Tooth germs after 2 days (T2) in culture. The teeth have reached the cap stage. (D, J) Tooth germs after 4 days (T4) in culture. The teeth have reached the early bell stage. (E,K) Sections through tooth germs cultured for 4 days. (A) A merged image of two images showing the pattern of *Tbx1* expression (a; red color) and the site of DiI injection (b; green color and arrow) in an E13.5 tooth bud. The superposition of the red and green colors (yellow color) indicates *Tbx1* expressing cells that were injected with DiI. (B) A DiI labeled spot at the tip of the bud near the epithelial–mesenchymal boundary (arrow). (C) Position of epithelial cells labeled with DiI (white arrow) at the cap stage. (D) Cells of the inner dental epithelium labeled with DiI (arrow) at the early bell stage. (E) Section showing labeling of cells of the inner dental epithelium (arrow). (F) *Tbx1* expression (violet color) in cells of the inner dental epithelium (arrowhead) during the early bell stage (E17.5). Arrow indicates the equivalent area of the bell-staged molar that was injected with DiI. (G) Superposition of two images showing the pattern of *Tbx1* expression (a; red color) and the site of DiI injection (b; green color and asterisk) in an E13.5 tooth bud. No yellow color is observed after the merging of the images. (H) DiI labeling in the center and left side (red spot; asterisk) of the bud. (I) DiI labeled cells in the center (asterisk) and left side (arrow) of the cap-staged tooth. No labeling is observed in the developing inner dental epithelium. (I) DiI labeling in the outer dental epithelium (arrow) and stellate reticulum (asterisk) of the bell-staged tooth. (K) Section showing labeling of cells of the outer dental epithelium (arrow) and stellate reticulum (asterisk). (L) *Tbx1* expression (violet color) in cells of the inner dental epithelium (arrowhead) during the early bell stage (E17.5). Arrow and asterisk indicate the equivalent areas of the bell-staged molar that were injected with DiI. Abbreviations: cm, condensing mesenchyme; de, dental epithelium; df, dental follicle; ide, inner dental epithelium; ode, outer dental epithelium; oe, oral epithelium; p, papilla; sr, stellate reticulum. Scale bars: 200 μm.

hybridization in 18 cultured homotypic and heterotypic tissue recombinants and the results obtained were constant according to the type of recombination.

In homotypic dental recombinants, *Tbx1* expression was observed in epithelial cells in contact with the mesenchyme (Fig. 3C). Similarly, *Tbx1* expression was induced in the epithelium when placed on top of the dental mesenchyme (Fig. 3F). However, when dental epithelial tissues were cultured alone, *Tbx1* transcripts were absent (Fig. 5G). To investigate whether epithelial *Tbx1* expression could be induced by any kind of mesenchyme, we examined *Tbx1* expression in heterotypic recombinants. When dental epithelium was recombined with an E12.5 non-dental mesenchyme, expression of the *Tbx1* gene was not observed in the epithelium after 24 h in culture (Fig. 3D), indicating that dental mesenchyme-derived signals are required to induce and/or maintain *Tbx1* expression in the epithelium. To investigate whether dental mesenchyme is sufficient to induce *Tbx1* expression in any

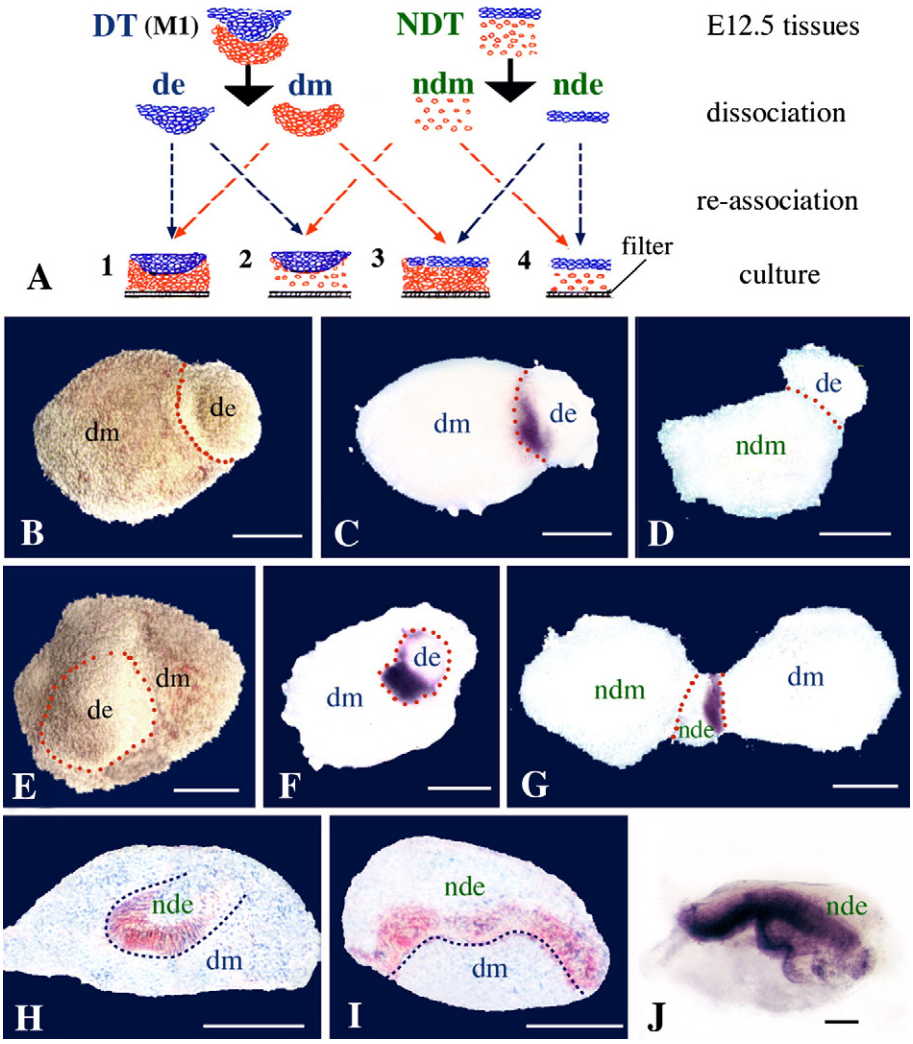

Fig. 3. Localization of *Tbx1* transcripts in explants of recombined E12.5 epithelium and mesenchyme from dental (DT) and non-dental tissues (NDT). Whole mount in situ hybridization (C, D, F, G, J) and in situ hybridization on sections (H, I) using the digoxigenin-labelled *Tbx1* probe is shown. (A) Schematic representation of the experimental plan. (B) Explants of recombined dental epithelium (de) and dental mesenchyme (dm) after 24 h in culture. The dotted lines represent the borders between the epithelial and mesenchymal tissues. *Tbx1* transcripts (violet color) in epithelial cells. (C) Explants of recombined dental epithelium and dental mesenchyme after 24 h in culture. *Tbx1* expression in epithelial cells. (D) Explants of recombined dental epithelium and non-dental mesenchyme (ndm) after 24 h in culture. *Tbx1* transcripts are absent from dental epithelium. (E) A dental epithelium cultured on top of a dental mesenchyme for 24 h. (F) *Tbx1* mRNA in the epithelium. (G) A non-dental epithelium (nde) cultured as a sandwich together with a dental and a non-dental mesenchyme. *Tbx1* expression in epithelium contacting the dental mesenchyme. (H, I) *Tbx1* transcripts (red color) in epithelial cells in explants of recombined non-dental epithelium and dental mesenchyme after 3 (A) and 5 (B) days in culture. Note the bud (H) and cap (I) configuration of the epithelium. (J) Expression of *Tbx1* in a bell-staged epithelium in recombinants of a non-dental epithelium and a dental mesenchyme after 7 days in culture and dissociation from the underlying mesenchyme. Scale bars: 200 μm.

epithelium, we combined E12.5 dental mesenchyme with E12.5 non-dental epithelium (palate, lip). After 24 h in culture, *Tbx1* transcripts were found in the epithelial cells in contact with the dental mesenchyme, but not in those combined with non-dental mesenchyme (Fig. 3G), indicating that dental mesenchyme has the capacity to ectopically induce *Tbx1* expression. When E12.5 dental mesenchymal tissues were cultured together with E12.5 non-dental epithelia for longer periods of time (3 to 7 days), the epithelia acquired the dental bud (Fig. 3H), cap (Fig. 3I) and bell (Fig. 3J) configurations and expressed *Tbx1* in cells contacting the dental mesenchyme (Figs. 3H–J).

FGFs can mimic mesenchymal signals that are responsible for Tbx1 expression in epithelium

We next attempted to elucidate the mesenchymal signal that is responsible for the induction/maintenance of *Tbx1* in epithelium. Both BMPs and FGFs molecules are important for tooth initiation and morphogenesis and therefore might regulate *Tbx1* expression in dental epithelium, as reported in other tissue/organ systems during development (Vitelli et al., 2002; Bachiller et al., 2003). To test this hypothesis, affi-gel agarose beads loaded with either BMP2 or BMP4 (250 μg/ml) and heparin acrylic beads loaded with either FGF2 or FGF3 or FGF4 (100 μg/ml) were placed either on top of dissected E12.5 mandibles at the sites where teeth develop (Figs. 4A–F) or on top of 14 isolated E12.5 dental epithelial explants (Figs. 4G–K). In mandibular explants, expression of *Tbx1* was upregulated by FGF2-releasing beads (Fig. 4D), while BMP4-releasing beads downregulated *Tbx1* expression in dental epithelium (Fig. 4E). *Tbx1* expression was not altered after implantation of control beads (Fig. 4F). In dental epithelial explants, *Tbx1* expression was observed in cells surrounding the FGF beads (Figs. 4H, I), but not in cells surrounding the BMP beads (Fig. 4J). *Tbx1* transcripts were absent from epithelial cells surrounding the control beads (Fig. 4K). These results suggest that either the mesenchymal signal for *Tbx1* maintenance in epithelium is a FGF molecule, or, alternatively, FGFs can mimic the signal emanating from the mesenchyme.

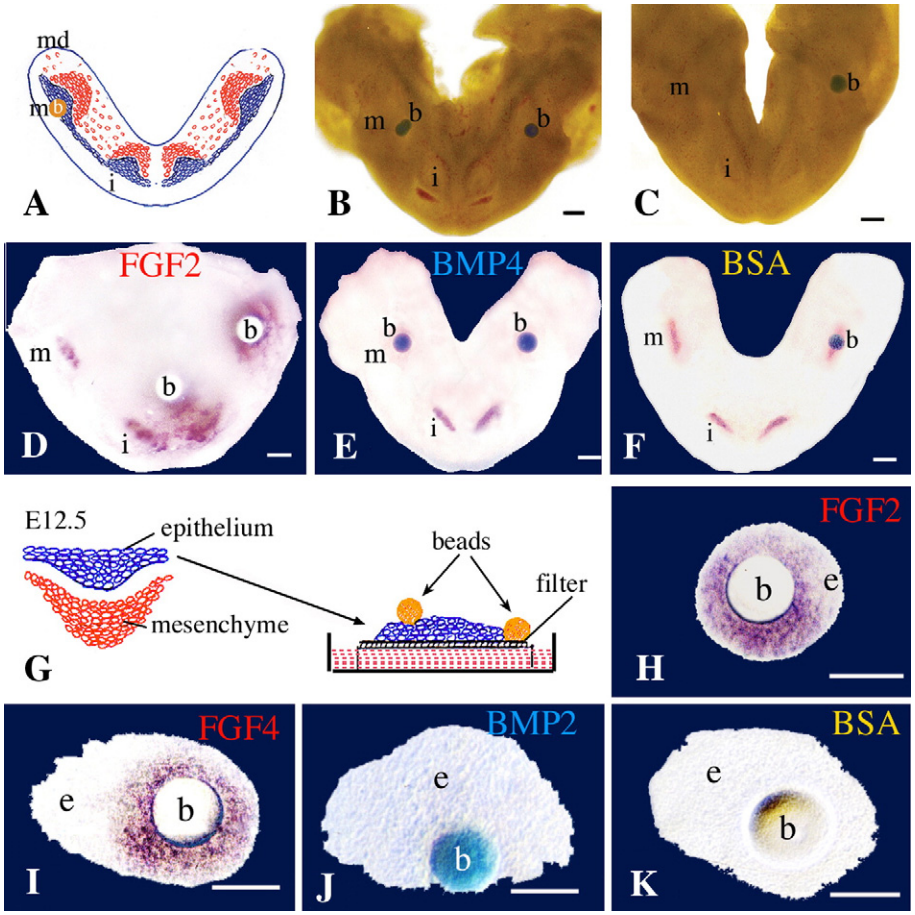

Fig. 4. Effects of FGF and BMP molecules on *Tbx1* expression in E12.5 mandible and dental epithelial explants cultured in vitro. Explants cultured together with beads loaded with FGF2 (D, H), FGF4 (I), BMP4 (E), BMP2 (J), and BSA (F, K). (A) Schematic representation of a mandible (md) cultured together with a bead (b). (B) Oral view of a mandible cultured together with BMP beads (blue color). (C) A mandible cultured together with a BSA control bead. (D) Upregulation of *Tbx1* expression in epithelium surrounding FGF2 beads (white color). (E) Downregulation of *Tbx1* expression by BMP4 beads. (F) Control beads do not alter *Tbx1* expression in molar (m). (G) Schematic representation of the experimental procedure used for the culturing of dental epithelium. (H, I) *Tbx1* expression in epithelium around the FGF-releasing beads. (J) *Tbx1* transcripts are absent from epithelial cells surrounding a BMP2-bead. (K) Control BSA-beads do not affect *Tbx1* expression. Additional abbreviations: e, epithelium; i, incisor. Scale bars: 200 μm.

Tbx1 is misexpressed in Fgfr2b$^{-/-}$ mice

Since FGF molecules control *Tbx1* expression in dental epithelial explants in vitro, we therefore studied the expression of *Tbx1* in developing teeth where FGF signaling is disrupted. During the initiation and early bud stages, FGF molecules signal through the FGF receptor Fgfr2b. The receptor is expressed in cells of the dental epithelium (Kettunen et al., 1998), which also express *Tbx1*. In Fgfr2b$^{-/-}$ mouse embryos, molars fail to progress beyond an early bud stage of development (De Moerlooze et al., 2000). *Tbx1* expression is downregulated in dental epithelium of E11.5 and E13.5 Fgfr2b$^{-/-}$ mice (Figs. 5B, D, F) when compared to wild-type littermates (Figs. 5A, C, E), thus confirming that FGF molecules interact with *Tbx1* during tooth morphogenesis.

Tbx1 activates amelogenin expression in oral epithelium

In order to address the function of *Tbx1*, we misexpressed it in oral epithelium using electroporation (Figs. 6A, C). For this purpose twelve E11.5 mandibles were collected and preceded for electroporation. Electroporation with a full-length human *TBX1* expression construct was satisfactory in four of the ten mandibles. In situ hybridization showed that *amelogenin* expression was induced in the epithelium at the sites of electroporation (Fig. 6E) of all four mandibles. Therefore, high-level *TBX1* transcription is able to induce *amelogenin* in the epithelium. Electroporation of a control GFP construct alone into the epithelium of two mandibles had no effect upon endogenous *Tbx1* and *amelogenin* expression (Figs. 6B, D, F).

Correlation of Tbx1 expression with cell proliferation in dental epithelium

In an attempt to ascertain whether expression of *Tbx1* is correlated with cell proliferation in the developing teeth, pregnant mice were injected with BrdU and tooth germs of E13 embryos were analyzed in parallel for *Tbx1* expression and cell proliferation. In E13 dental epithelium, territories of *Tbx1* expression (Fig. 7A; violet color) and cell proliferation (Fig. 7A; red color) are considerably overlapping; proliferation is also observed in the mesenchyme. We wanted then to test if this was also true in vitro. When E13 epithelium and mesenchyme were recombined, both proliferation and *Tbx1* transcripts were observed in epithelial cells contacting the mesenchyme (Fig. 7B). Similarly, induction of *Tbx1* expression in dental epithelium by FGF-releasing beads was correlated with increased cell proliferation around the beads (Fig. 7C).

Discussion

Direct evidence for a role of the T-box transcription factors in facial and tooth formation comes from the effect of mutations in human *TBX* genes (reviewed by Naiche et al., 2005). Mutations in *TBX3* cause a pleiotrophic disorder affecting, among other processes, tooth devel-

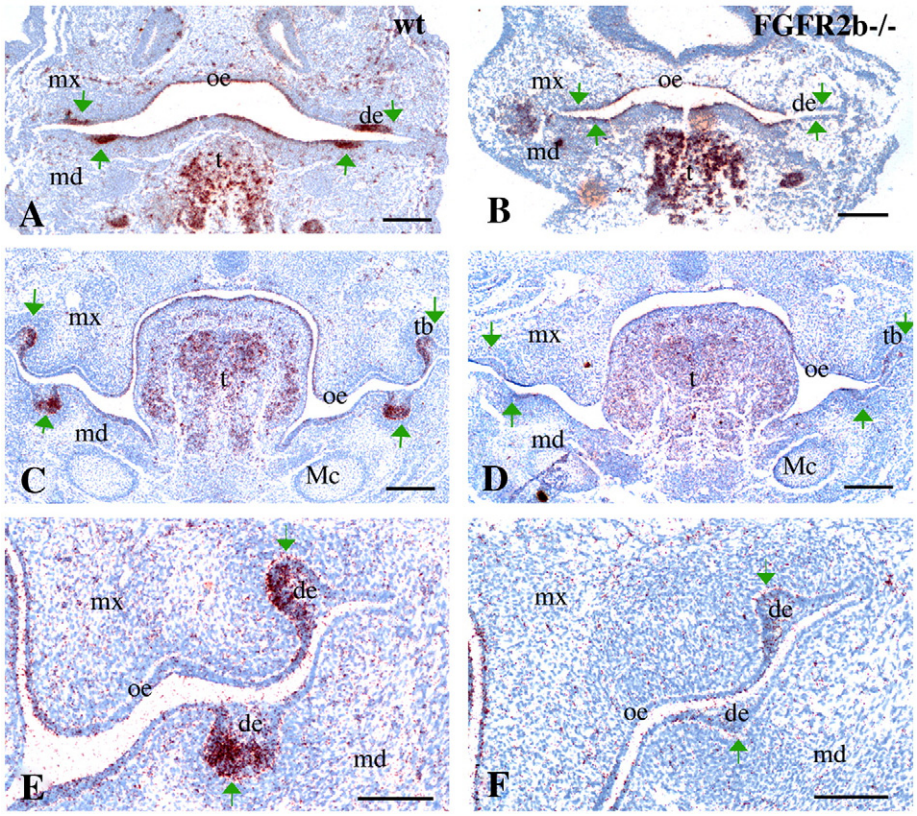

Fig. 5. Tbx1 expression is altered in dental epithelium of Fgfr2b$^{-/-}$ mice. ^{35}S-labelled in situ hybridization to detect *Tbx1* mRNA. Frontal tissue sections through the oral cavity of E11.5 (A, B) and E13.5 (C–F), wild-type (wt) (A, C, E) and *Fgfr2b*$^{-/-}$(B, D, F) mice. The molar teeth of *Fgfr2b*$^{-/-}$ mice (green arrows) fail to progress beyond an early bud stage of development (D, F). (A, C, E) *Tbx1* expression in dental epithelium (de; green arrows) of wild-type mice. (B, D, F) Downregulation of *Tbx1* expression in dental epithelium (green arrows) of *Fgfr2b*$^{-/-}$ mice. Additional abbreviations: Mc, Meckel's cartilage; md, mandibular process; mx, maxillary process; oe, oral epithelium; t, tongue; tb, tooth bud. Scale bars: 200 μm.

opment (Bamshad et al., 1997; Meneghini et al., 2006). *TBX1* is a candidate for the 22q11 deletion syndrome (22q11DS), which is a relatively common developmental anomaly that has been recognized as DiGeorge syndrome (DGS) or velocardiofacial syndrome (Chieffo et al., 1997; Jerome and Papaioannou, 2001; Lindsay et al., 2001). Subjects with 22q11DS display a variety of clinical manifestations including malformations within the craniofacial region such as face abnormality, mandibular retrognathia and cleft palate (Hammond et al., 2005). Several clinical studies on teeth of DGS patients have reported on the presence of hypodontia and enamel defects that range from hypoplasia to a generalized hypomineralization (Børglum-Jensen et al., 1983; Fukui et al., 2000). These anomalies have been attributed to hypocalcemia seen in 22q11DS patients (Fukui et al., 2000), but the tight *Tbx1* expression in cells destined to form enamel (i.e. inner dental epithelium, preameloblasts) suggests that the enamel defects could be linked to a TBX1 deficiency. Striking facial and odontogenic defects have been also observed in mutant mice lacking the *Tbx1* gene (Jerome and Papaioannou, 2001). These mice exhibit cleft palate and hypoplastic maxillary incisors, but a detailed description of the tooth phenotype is missing because these mice die perinatally (Jerome and Papaioannou, 2001). These clinical and genetic findings indicate that Tbx1 plays a significant role in mediating the complex signaling interactions that occur during odontogenesis for the determination, differentiation and correct function of ameloblasts.

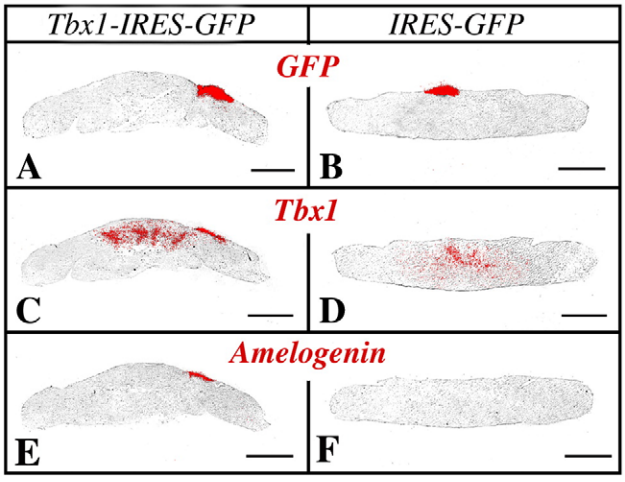

Fig. 6. Electroporation of *TBX1-IRES-GFP* (A, C, E) or control *IRES-GFP* (B, D, F) constructs into the epithelium of E11.5 mandibular explants. (A, B) *GFP* expression marking the site of electroporation. (C, D) *Tbx1* expression is observed only in the epithelium of the electroporated with *TBX1-IRES-GFP* mandibular explant (C), while epithelial expression is not detected in explants electroporated with IRES-GFP (D). Note the endogenous *Tbx1* expression in the underlying mesenchyme of both experimental and control cultures (C, D). (E, F) Upregulation of *Amelogenin* expression only in the epithelium of explants electroporated with *TBX1-IRES-GFP* (E). Scale bars: 200 μm.

Transcription factors and signaling molecules are involved in the determination and differentiation of specific cell populations within dental tissues. During tooth formation, a subpopulation of oral epithelial cells acquires odontogenic potential and progressively forms a complex structure of four cell layers (i.e. stratum intermedium, stellate reticulum, outer and inner dental epithelium), known collectively as the enamel organ. Cells of the inner dental epithelium undergo a precise developmental program resulting in their differentiation into ameloblasts and the expression of specific gene products forming the enamel matrix (Zeichner-David et al., 1995). The temporospatial behavior of dental epithelial cells during

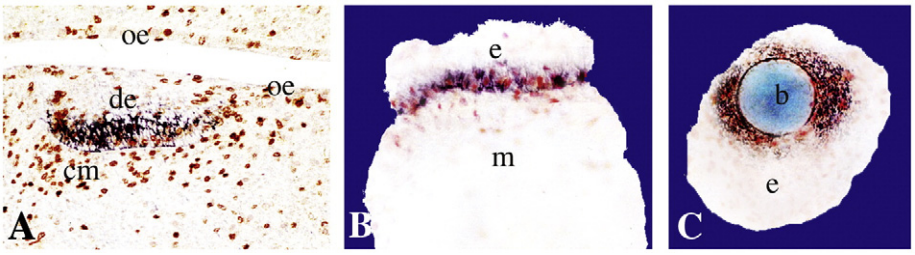

Fig. 7. Correlation of *Tbx1* expression and cell proliferation. In situ hybridization using a digoxigenin-labeled *Tbx1* probe and anti-BrdU immunohistochemistry. (A) *Tbx1* expression (violet color) and cell proliferation (nuclei in red) in the epithelium of an E13 molar tooth. (B) *Tbx1* expression (violet) in proliferating epithelial cells (red) adjacent to the recombined mesenchyme in an E13 dental explant cultured in vitro. (C) *Tbx1* expression in epithelial proliferating cells around a FGF3 releasing bead in an E13 dental epithelial explant. Abbreviations: b, bead; c, condensed mesenchyme; de, dental epithelium; e, epithelium; m, mesenchyme; oe, oral epithelium. Scale bars: 200 μm.

odontogenesis is not yet well known. Earlier studies using the mandible slice culture method have focused exclusively on the fate and migration of cells of the enamel knot (Matalova et al., 2005; Cho et al., 2007). The tooth buds develop at a rate much more similar to that observed *in vivo* in these cultures. This is presumably due to the greater access of the tooth germ to the culture medium. However, the slicing of the tissue will lead to some healing of the sectioned surface, but this does not appear to affect the morphology of the developing tooth germ. Gene expression patterns are also maintained in the developing tooth germs that were previously sectioned (Cho et al., 2007). We used the mandible slice culture method to show that there is no cellular continuity between the different cell precursors that give rise to the four dental epithelial cell layers. DiI labeling of the basal part of the epithelial bud shows that cells do not move out of this region. Similarly, cells of the median part do not intermingle with cells located elsewhere. Cells originated from the basal part are found in the inner dental epithelium, whereas cells from the median part are localized in the stellate reticulum. These results suggest that basal dental cells expressing *Tbx1* are the progenitors for cells of the inner dental epithelium. However, although the four dental epithelial layers appear to be originated from different cell populations, this does not mean that they can act independently. For example, regulation of proliferation and/or differentiation of inner dental epithelial cells may be directed by signals emanating from the other cell layers.

Ameloblast differentiation and enamel formation are tightly regulated events that occur during the late stages of odontogenesis. Amelogenin accounts approximately 90% of the proteins that are secreted by mature ameloblasts and play a major role in the biomineralization and structural organization of enamel (Zeichner-David et al., 1997). *In vitro* experiments have shown that the dental epithelium is capable of expressing the enamel matrix proteins amelogenin and tuftelin already at E13 (Couwenhoven and Snead, 1994; Zeicher-David et al., 1995), much earlier than the start of cytodifferentiation and mineralization events. These studies have also shown that *amelogenin* is expressed in cultured E14 dental epithelia (cap stage), but not in the bud-staged E12–E13 epithelia (Couwenhoven and Snead, 1994). The prolonged culture of the E12–E13 epithelia has failed to induce detectable levels of *amelogenin* (Couwenhoven and Snead, 1994). These results suggest that the instructive signals, which control transcription of the enamel matrix proteins, occur during the bud stage, and, furthermore, indicate that ameloblast determination begins in early progenitors that represent a small proportion of dental epithelial cells. However, transcriptional regulators that distinguish inner dental epithelium from the rest of dental epithelium at such early stages have not yet been identified. During the bud stage, several transcription factors such as *Pitx1*, *Pitx2*, *Islet1* (Mitsiadis et al., 2003; Mitsiadis and Drouin, 2008; Mucchielli et al., 1997) are specifically expressed in the dental epithelium, suggesting that these molecules could be regulators of *amelogenin* expression. Here we show that *Tbx1* is expressed in dental epithelial cells as early as E12.5 (bud stage) and progressively its expression become restricted to cells of the inner dental epithelium. These cells that are mitotically active and morphologically indistinguishable from other immature dental epithelial cells will differentiate into ameloblasts during the late bell stage. Tbx1 acts as a direct or indirect regulator of *amelogenin* expression on dental epithelial cells since forced *Tbx1* expression in oral epithelium is able to induce *amelogenin* transcription. The amelogenin protein has been initially detected in dental tissues at E18.5 (Zeichner-David et al., 1997), but more recent studies have shown that the protein is expressed in tooth germs at E13.5, and reaches high levels of expression at E18.5 (Gruenbaum-Cohen et al., 2007). Its early expression raises the possibility of additional functions for amelogenin such as to act as a signaling molecule during early stages of tooth development. Indeed, bead implantation experiments in E13.5 dental tissues using human recombinant amelogenin protein ($rHAM^{+}$) have shown that amelogenin is involved in the recruitment of mesenchymal cells (Gruenbaum-Cohen et al., 2007; personal communication). Taken together these results suggest that the fate of dental epithelial cells is determined very early during embryogenesis, and that inner dental epithelial cells may exist in a protodifferentiated state, which is characterized by the concomitant expression of *Tbx1* and *amelogenin*.

Expression of *Tbx1* in dental epithelium could be activated/maintained by signals originated from either the epithelium or the mesenchyme or from both tissues. Tissue recombination experiments have shown that the source of these signals is the underlying dental mesenchyme, for the following two reasons: firstly, *Tbx1* expression in the dental epithelium is downregulated in recombinants with non-dental mesenchyme; secondly, the E12.5 dental mesenchyme induces *Tbx1* expression in non-dental (*Tbx1*-negative) epithelium. Hence, dental mesenchyme is able to induce and/or maintain *Tbx1* expression in epithelium. Several FGF molecules such as FGF2, FGF3 and FGF10 are

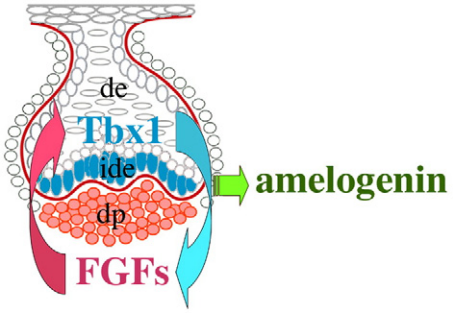

Fig. 8. Schematic representation of a model illustrating the regulatory loop between Tbx1 and FGF molecules in dental tissues. FGFs and signals derived from the dental papilla mesenchyme (red color; dp) are responsible for activation and/or maintenance of *Tbx1* expression in cells of the inner dental epithelium (blue color; ide). Similarly, expression of FGFs is dependent on Tbx1 signaling. In dental epithelium (de) Tbx1 induces amelogenin expression, and, in combination with FGF molecules, controls the proliferation and survival of the ameloblast precursors (i.e. ide cells).

expressed in dental mesenchyme during the bud and cap stages of tooth morphogenesis (Cam et al., 1992; Harada et al., 2002; Kettunen et al., 2000). Uniquely among the FGF receptors, Fgfr2b is expressed during the early stages of tooth development, showing an exclusive epithelial expression pattern (Kettunen et al., 1998). Thus, FGF molecules expressed in dental mesenchyme may act in a paracrine manner to affect cell behavior and *Tbx1* expression in dental epithelium. Indeed, the implantation of beads loaded with FGFs resulted in cell proliferation and the concomitant *Tbx1* upregulation in cultured E12.5 dental epithelia. A close correlation between *Tbx1* expression and cell proliferation also exists in dental epithelium *in vivo*. The T-box transcription factors are important for the control of cell proliferation in various tissues and organs (Hatcher et al., 2001), and thus Tbx1, in combination with FGFs, may act as a survival factor stimulating the proliferation of inner dental epithelial cells. FGF molecules may have redundant functions during epithelial tooth morphogenesis. This is supported by previous findings showing arrest of tooth development at the bud stage in Fgfr2b deficient mice (De Moerlooze et al., 2000), but no tooth arrest in FGF3 and FGF10 knockout mice (Mansour et al., 1993; Min et al., 1998; Sekine et al., 1999; Ohuchi et al., 2000). FGF3$^{-/-}$ mice have defective enamel and compound FGF3$^{-/-}$ and FGF10+/−mutant mice have very thin or no enamel supporting the idea that these genes control the proliferation of ameloblast precursors (Harada et al., 2002; Wang et al., 2007). FGF molecules genetically interact with Tbx1 and it is possible that they form a regulatory loop in teeth since the expression of FGFs (i.e. FGF3, FGF8, FGF10) is down regulated in the Tbx1$^{-/-}$ mutants (Aggarwal et al., 2006; Hu et al., 2004; Viteli et al., 2002) and the expression of Tbx1 is considerably diminished in the dental epithelium of the Fgfrb2 mutant mice. A regulatory relationship between the *T-box* genes and FGFs has been already described in other organs of various species (Griffin et al., 1995; Logan et al., 1998; Viteli et al., 2002). A role for Tbx1 in the regulation of FGFs within the dental tissues could result in a failure to form and/or maintain the necessary number of ameloblast precursors that could explain the resulting hypoplastic phenotype in the incisors of the Tbx1$^{-/-}$ mice (Jerome and Papaioannou, 2001). Additional mesenchyme-derived signals are needed at more advanced developmental stages (i.e. late bell stage) to induce cells of the inner dental epithelium to withdraw from mitosis, differentiate into ameloblasts, and express high levels of amelogenin.

In conclusion, the present data show that mesenchyme-derived signals and FGF molecules maintain epithelial *Tbx1* expression in developing teeth. Tbx1 and FGFs form a regulatory loop that is important for the specification, proliferation and survival of the ameloblast progenitors (Fig. 8). Further, Tbx1 is one of the direct or indirect signals that are required for the initiation of *amelogenin* expression in dental tissues. Tbx1 may therefore represent a potential marker for presumptive ameloblasts.

Acknowledgments

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